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Mutations in NS5B Polymerase of Hepatitis C Virus: Impacts on *in Vitro* Enzymatic Activity and Viral RNA Replication in the Subgenomic Replicon Cell Culture

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Hepatitis C virus (HCV) nonstructural protein 5B (NS5B) is an RNA-dependent RNA polymerase (RdRp) essential for virus replication. Several consensus sequence motifs have been identified in NS5B, some of which have been shown to be critical for its enzymatic activity. A unique β-hairpin structure located between amino acids 443 and 454 in the thumb subdomain has also been shown to play an important role in ensuring terminal initiation of RNA synthesis *in vitro*. However, the importance of these sequence and structural elements in viral RNA replication in infected cells has not been established, mainly due to the lack of a reliable cell culture system for HCV. In this study, we investigated the effect of several single amino acid substitutions and β-hairpin truncations in NS5B on viral RNA replication by using the subgenomic replicon cell culture system. A strong correlation between *in vitro* polymerase activity and viral RNA replication was observed with most of the substitutions. Interestingly, truncations of the β-hairpin (by four and eight amino acid residues, respectively), which did not reduce the *in vitro* enzymatic activity, completely abolished the ability of the replicon RNA to replicate in Huh-7 cells, demonstrating its essential role in viral RNA replication. Furthermore, a conservative substitution in motif D, from an arginine residue (AMT$\underline{R}^{345}$), which is conserved among all HCV isolates, to a lysine residue, resulted in significant improvements in both transient RNA replication and colony formation efficiencies. This result also correlates with a previous observation that the enzymatic activity of NS5B increased by about 50% when the same NS5B substitution was introduced (V. Lohmann, F. Korner, U. Herian, and R. Bartenschlager, *J. Virol.* 1997, 71, 8416–8428).

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of non-A, non-B transfusion-associated hepatitis and accounts for a significant proportion of hepatitis cases worldwide (Houghton, 1996; Rice, 1996). Although HCV infection resolves in some cases, the virus establishes chronic infection in up to 80% of the infected individuals and persists for decades. Recent studies of the natural history of HCV infection indicate that the majority of people with chronic HCV infection have relatively mild disease with slow progression. However, an estimated 20% of these infected individuals will go on to develop cirrhosis and 1 to 5% will develop liver failure and hepatocellular carcinoma (Saito *et al.,* 1990; Seeff, 1999; World Health Organization, 1996). It is estimated that chronic HCV infection affects more than 4 million people in the United States, 8 million in Europe, and 170 million worldwide. Chronic hepatitis C infection is the leading cause of chronic liver disease and the leading indication for liver transplantation in the United States. The Centers for Disease Control and Prevention estimates that hepatitis C currently is responsible for approximately 8000 to 10,000 deaths in the United States annually.

HCV is a positive-sense RNA virus belonging to the *Flaviviridae* family (Choo *et al.,* 1989). The life cycle of HCV consists of several important processes that occur primarily in the cytoplasm of the host cells. Among these intricate processes, RNA replication is the centerpiece of the viral proliferation cycle. While many viral and/or host factors are suspected to be involved, one certainty is that the virally encoded polymerase controls the entire replication process. The genome of HCV consists of approximately 9600 bases with a single open reading frame (ORF) encoding a polyprotein of around 3010 amino acids. The ORF is flanked by 5′ and 3′ untranslated regions (UTRs) of several hundred nucleotides, which are important for RNA translation and/or replication. The polyprotein is cleaved both co- and post-translationally by the cellular and virally encoded proteases into at least 10 separate mature viral structural and nonstructural proteins, from the amino- to the carboxyl-terminus: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Bartenschlager and Lohmann, 2000; Houghton, 1996; Rice, 1996). NS5B is the virally encoded RNA-dependent RNA polymerase (RdRp) which consists of three typical subdomains: finger, palm, and thumb. Several conserved sequence motifs have been proposed in the palm (motifs A, B, C, D, E) and finger subdomain (motif F) (Ago *et al.,* 1999; Behrens *et al.,* 1996; Bressanelli *et al.,* 1999; De Francesco *et al.,* 1996; Ferrari *et al.,* 1999; Lesburg *et al.,* 1999; Loh-

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mann *et al.,* 1997, 1998). The enzymatic activity of NS5B has been characterized in several studies and a limited mutational analysis was performed which confirmed the requirement of several sequence motifs in the palm subdomain for polymerase activity (Behrens *et al.,* 1996; De Francesco *et al.,* 1996; Ferrari *et al.,* 1999; Hong *et al.,* 2001; Lohmann *et al.,* 1997, 1998; Luo *et al.,* 2000; Oh *et al.,* 1999, 2000; Sun *et al.,* 2000; Zhong *et al.,* 2000a,b). Structural comparison between HCV NS5B and poliovirus $3D^{pol}$ also revealed a unique structural motif (β-hairpin) in the thumb subdomain of HCV NS5B that consists of 12 amino acids (^{443}LDCQIYGACYSI^{454}) (Ago *et al.,* 1999; Bressanelli *et al.,* 1999; Lesburg *et al.,* 1999). Our previous result suggested that HCV NS5B may use this β-hairpin to ensure terminal initiation of RNA synthesis by correctly positioning the 3′ terminus of viral RNA at the active site, as truncation of the β-hairpin resulted in RNA synthesis initiating from internal positions of the template RNA (Hong *et al.,* 2001). The ability to initiate RNA synthesis from the 3′ terminus of viral RNA is believed to be critical for retaining all genetic/sequence information during viral RNA replication directed by an RdRp that uses a *de novo* mechanism for initiation.

In the past, molecular analyses of HCV replication have been hindered by the lack of a robust, reproducible cell culture system permissive to HCV replication *in vitro* and the lack of a manageable small animal model other than the chimpanzee. Despite the success in obtaining infectious HCV cDNA clones that replicate in chimpanzee (Beard *et al.,* 1999; Hong *et al.,* 1999; Kolykhalov *et al.,* 1997; Yanagi *et al.,* 1997, 1998), efforts to establish a tissue culture replication system have proven to be rather problematic. This situation has been alleviated recently with the development of the HCV subgenomic replicon system (genotype 1b) in human hepatoma cell line human hepatoma cell line (Huh-7) (Blight *et al.,* 2000; Krieger *et al.,* 2001; Lohmann *et al.,* 1999, 2001; Pietschmann *et al.,* 2001). In this system, the HCV structural genes (C, E1, and E2) were replaced with a selection marker, the neomycin phosphotransferase gene, and the translation of nonstructural genes was driven by the IRES element from encephalomyocarditis virus (EMCV). When the subgenomic replicon RNA was transfected into Huh-7 cells, colonies resistant to G418 selection were generated, though at very low frequency. Some of the neomycin-resistant cell lines carried the self-replicating HCV RNAs as high as several thousand copies per cell (Lohmann *et al.,* 1999). More recently, adapted mutations that significantly improved the transduction efficiency of the original replicon have been identified (Blight *et al.,* 2000; Lohmann *et al.,* 2001; Pietschmann *et al.,* 2001). The exact mechanism by which these cell culture adaptations occur is not clear. Nonetheless, establishment of a reliable cell-based replication system makes the genetic and functional analyses of HCV replication feasible.

In this study, we engineered a number of single residue substitutions and β-hairpin truncations in HCV NS5B and analyzed their effects on *in vitro* polymerase activity and viral RNA replication in the replicon cell culture system. A strong correlation between enzymatic activity and replicon RNA replication was observed with most of the mutations. Interestingly, truncations of the β-hairpin completely abolished the ability of the replicon RNA to replicate in the transfected cells, despite the fact that the enzymatic activity of the truncated NS5B was clearly enhanced in both primer-dependent and *de novo* initiation assays. Additionally, a conservative substitution in motif D (AMT$\underline{\text{R}}^{345}$), from an arginine residue (R), which is conserved in all HCV isolates, to a lysine residue (K) that is present at this position in all other RdRps and RTs, significantly improved the replication efficiency of the replicon RNA. Therefore, conservation of the less optimal or inhibitory arginine residue in NS5B for reducing viral RNA replication may have potential implications in HCV propagation and establishment of persistent infection *in vivo*.

RESULTS

Engineering of single amino acid substitutions and β-hairpin truncations in NS5B

Amino acid sequence alignments between HCV NS5B and other flavivirus RdRps have revealed six conserved sequence motifs (A, B, C, D, E, and F) characteristic of all RdRps (Behrens *et al.,* 1996; De Francesco *et al.,* 1996; Ferrari *et al.,* 1999; Lesburg *et al.,* 1999; Lohmann *et al.,* 1997, 1998). Motif A is involved in magnesium coordination and possibly ribonucleotide selection. Motif B has also been implicated in ribonucleotide selection. Motif C is the catalytic motif that coordinates magnesium binding and catalyzes the nucleotidyl transfer reaction. The exact function of motif D is not clear. Only one position, a positively charged residue, seemed to be maintained among all RdRps. This position is a lysine in all reverse transcriptases (RTs) and nearly all RdRps. However, an arginine residue is found at this position in all HCV isolates. Motif E is located at the junction between palm and thumb subdomains and is unique to RdRps and RTs. This motif has been proposed to interact with the primer strand and has been referred to as the "primer grip." Motif F is a newly identified motif in the finger subdomain containing several positively charged residues (Lesburg *et al.,* 1999). It is predicted to be involved in interaction(s) with RNA template, primer, or NTP. In addition, three-dimensional structure of HCV NS5B also revealed a unique β-hairpin structure in the thumb subdomain (Ago *et al.,* 1999; Bressanelli *et al.,* 1999; Lesburg *et al.,* 1999). This β-hairpin is absent in poliovirus $3D^{pol}$ RdRp and HIV RT. Our previous study showed that this β-hairpin may play an important role in ensuring initiation of RNA synthesis from the 3′ terminus of the template RNA, a function that correlates closely with the *de novo* initiation of RNA replication by HCV NS5B (Hong *et al.,* 2001).

We focused our study on a number of residues in the

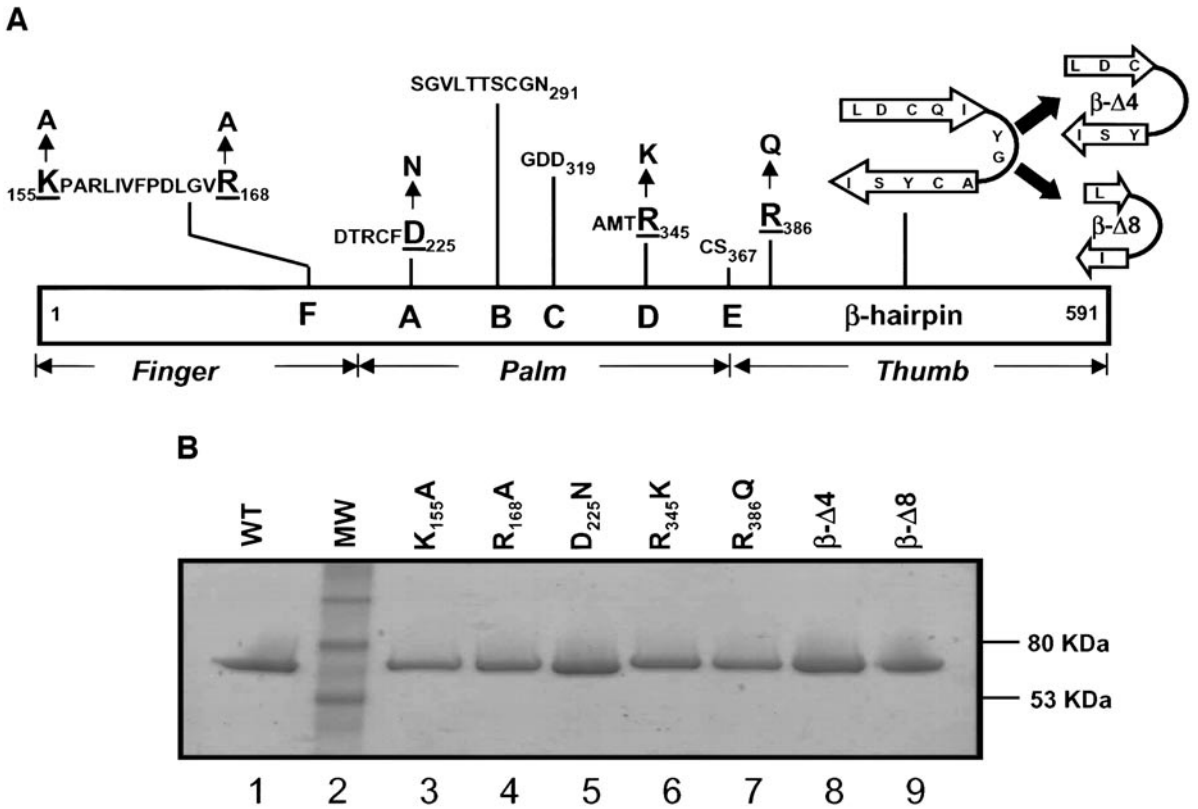

FIG. 1. Purification of HCV NS5B proteins containing single residue substitutions and β-hairpin truncations. (A) Schematic diagram showing the conserved sequence motifs and β-hairpin in HCV NS5B. Individual substitutions and β-hairpin truncations are indicated. (B) Purification of NS5B proteins. Recombinant NS5B proteins containing the required modifications were expressed in *E. coli* with a C-terminal 21 amino acid deletion and were purified to homogeneity as previously reported (Ferrari *et al.,* 1999; Lesburg *et al.,* 1999). About 2 μg of each protein was analyzed on an SDS–PAGE gel. The slight difference in gel mobility may reflect the combined effect of amino acid changes in the NS5B protein and the amounts of protein loaded.

conserved sequence motifs and the unique β-hairpin structure to analyze their requirements for *in vitro* polymerase activity and for viral RNA replication in the subgenomic replicon system (Huh-7 cells) (Blight *et al.,* 2000; Lohmann *et al.,* 1999). The goal of the study was to determine the correlation between NS5B enzymatic activity and replicon RNA replication in the cells. To this end, the following single amino acid substitutions and small truncations were introduced into NS5B (Fig. 1A): (i) Motif F: lysine at position 155 to alanine ($K_{155}A$); and arginine at 168 to alanine ($R_{168}A$). The function of these two positively charged residues has not been defined, but likely involves an interaction with RNA template, primer, and/or NTP (Lesburg *et al.,* 1999). (ii) Motif A: aspartate at 225 to asparagine ($D_{225}N$). This aspartate residue is believed to play a role in selection of ribose sugars, likely by forming a hydrogen–bond interaction with the 2′ hydroxyl group of the ribose. (iii) Motif D: the conserved arginine at 345 to lysine ($R_{345}K$). This substitution has been shown previously to enhance polymerase activity by about 50% in an *in vitro* enzymatic assay (Lohmann *et al.,* 1997). (iv) Arginine at 386 in the thumb subdomain to glutamine ($R_{386}Q$). A preliminary study suggested that this arginine may be involved in stabilizing the interaction between the initiation nucleotide/dinucleotide and the template RNA (Hong *et al.,* 2001). (v) β-hairpin: two small truncations, four and eight amino acids, respectively (β-Δ4 and β-Δ8), were introduced to shorten the length of the β-hairpin. The truncations were designed such that an equal number of residues were deleted from each strand of the hairpin to maintain the polarity of the β-hairpin.

The mutations were introduced into the NS5B gene of the HCV subgenomic replicon construct (pNK5.1) (Krieger *et al.,* 2001). The modified NS5B genes with a 21 amino acid deletion at the carboxyl-terminus were then cloned into a bacterial expression vector and expressed in bacteria. The proteins were purified as described previously (Fig. 1B) and subjected to *in vitro* polymerase assays.

Primer-dependent elongation and *de novo* initiation activity of the mutant NS5B proteins

Two assay formats were used to determine the polymerase activity of the various NS5B mutants in comparison to that of the wild-type NS5B. The first assay used

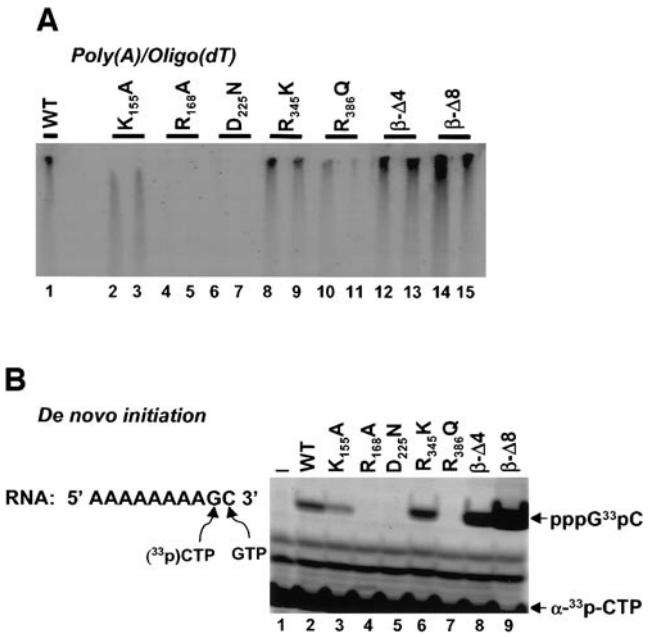

FIG. 2. Primer-dependent and *de novo* initiation activities of the modified NS5B proteins. (A) Primer-dependent polymerization assay. Poly(A) and oligo(dT) were used as the template and primer to direct incorporation of [α-^{33}P]UTP. Two independent preparations of each protein construct were tested along with the wild-type NS5B (lane 1). Reaction products were resolved on a 15% PAGE/6 M urea/TBE gel and analyzed by autoradiography. (B) *De novo* initiation assay. A synthetic RNA template (5′ AAAAAAAAGC 3′) was used in the presence of 100 μM GTP and [α-^{33}P]CTP for synthesis of dinucleotide product (pppG^{33}pC). Reaction products were separated on a 25% PAGE/6 M urea/TBE gel. The more active protein preparation for each construct (A) was used for the initiation assay. The corresponding polymerases were indicated on the top. Lane 1 is the reaction with no enzyme added.

poly(A) RNA as template and oligo(dT) as primer to measure the primer-dependent polymerization activity. Under these assay conditions, NS5B activity requires the presence of both poly(A) RNA and oligo(dT) primer (data not shown), which indicates a lack of significant nontemplated nucleotide addition. The second assay determined the *de novo* initiation activity of the polymerase by measuring the formation of dinucleotide product from a single initiation nucleotide. In this case, NS5B used a synthetic RNA (5′ AAAAAAAAGC 3′) as template to direct initiation of RNA synthesis from GTP (the initiating nucleotide) and CTP (the elongating nucleotide) and formation of the radiolabeled dinucleotide product (pppG^{33}pC). As shown in Fig. 2, R_{168}A and D_{225}N substitutions completely abolished both primer-dependent and *de novo* initiation activity (Fig. 2A, lanes 4/5 and 6/7; Fig. 2B, lanes 4 and 5). K_{155}A substitution drastically reduced both activities (Fig. 2A, lanes 2/3; Fig. 2B, lane 3). Interestingly, R_{386}Q substitution completely removed the *de novo* initiation activity (Fig. 2B, lane 7), but still retained low levels of the primer-dependent elongation activity (Fig. 2A, lane 10/11). This result is in line with the proposed function that R_{386} may play a role in stabilizing the interaction between the initiation NTP and the template RNA (Hong *et al.,* 2001), an interaction essential for *de novo* initiation of RNA synthesis. In contrast, the β-hairpin truncations did not abolish either polymerase activity. Instead, a significant enhancement of these activities over that of the wild-type protein was observed with β-Δ4 and β-Δ8, with β-Δ8 having the highest activity (Fig. 2A, lanes 12/13, 14/15; Fig. 2B, lanes 8 and 9). Finally, the conservative substitution (R_{345}K) in motif D also increased the RdRp activity by 20–50% over that of the wild-type NS5B in these assays (compare lanes 8 vs 1 in Fig. 2A, lanes 6 vs 2 in Fig. 2B). This observation is in agreement with a previous report that a ~50% enhancement of RdRp activity was observed by the same substitution *in vitro* (Lohmann *et al.,* 1997).

In addition to enhancing polymerase activity, β-hairpin truncations were also found to increase, in proportion to the length of the truncation, the ability of the polymerase to use pre-annealed, stable duplex RNA as template and to initiate RNA synthesis from internal positions of the template RNA as reported previously (Hong *et al.,* 2001). As shown in Fig. 3A, the ability of NS5B to use an end-labeled duplex RNA (P) as template to direct single nucleotide incorporation (product: P + 1) increased proportionally to the extent of the truncation (β-Δ8 > β-Δ4 > wild-type) (Fig. 3A, lanes 2–4). This result suggested that the presence of the β-hairpin may interfere with the docking of double-stranded RNA template onto the active site. Furthermore, upon truncation of the β-hairpin, NS5B became capable of initiating RNA synthesis from the internal position of a template RNA. Figure 3B shows the initiation assay in which a radiolabeled dinucleotide primer (^{33}pGpG) was used to prime initiation of RNA synthesis (Hong *et al.,* 2001; Zhong *et al.,* 2000a). Unlike the wild-type NS5B that only initiated RNA synthesis from the 3′ terminal dicytidylates (CC) of the template (lanes 1 vs 2), the β-hairpin truncation mutants were able to initiate RNA synthesis from both terminal and internal CC positions (lanes 3–6). This internal initiation activity was also in proportion to the extent of the truncation (β-Δ8 > β-Δ4 > wild-type) (compare lanes 2, 4, and 6). Collectively, these results suggest that the unique β-hairpin plays multiple roles in regulating viral polymerase activity. It is not clear, however, which of these potential functions is crucial for viral RNA replication in the infected cells.

Replication of HCV subgenomic replicon RNA carrying NS5B mutations in Huh-7 cells

After determination of the *in vitro* enzymatic activity, the NS5B mutations were engineered into the HCV subgenomic replicon clone pNK5.1 (an adapted version of the original replicon clone, pFK-I389neo/NS3–3′/wt) (Blight *et al.,* 2000; Krieger *et al.,* 2001; Lohmann *et al.,* 1999) through site-directed mutagenesis. The modified

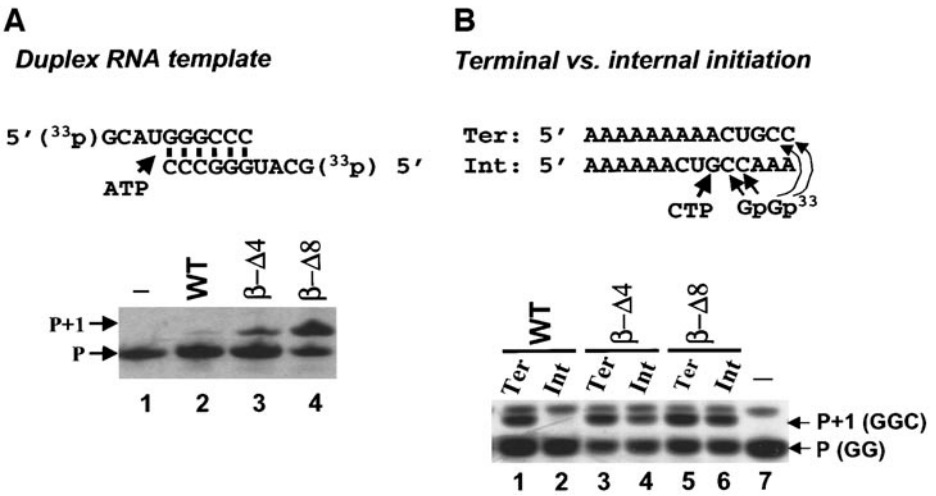

FIG. 3. Use of duplex RNA template and internal initiation of RNA synthesis by β-Δ4 and β-Δ8. (A) A pre-annealed, end-labeled double-stranded synthetic RNA was used as template by wild-type NS5B (lane 2), β-Δ4 (lane 3), and β-Δ8 (lane 4) to catalyze single nucleotide (AMP) incorporation (Hong *et al.,* 2001; Zhong *et al.,* 2000a). The bands representing the input template (P: 10-mer) and the elongated product (P + 1: 11-mer) are indicated. In lane 1, the reaction contained no enzyme. About 5 μM of the labeled duplex RNA, 100 μM ATP, and ~1 μg of each NS5B protein were used under the standard reaction conditions. Reaction products were resolved on a 20% PAGE/6 M urea/TBE gel and subjected to autoradiography. (B) Internal initiation assay. Two synthetic RNA templates (Ter and Int) with dicytidylate (CC) sequence either at the 3′ terminus (Ter) or an internal position (Int) were used as template along with the ^{33}pGpG dinucleotide primer (Zhong *et al.,* 2000a). Single nucleotide (CMP) incorporation was detected. The bands representing the labeled primer (P) and the elongated product (P + 1) are indicated. Lane 7 is the control reaction containing WT enzyme but no RNA template. About 5 μM of template RNA, 10 μM of dinucleotide primer, 100 μM CTP, and 1 μg of NS5B protein were used in each reaction. Reaction products were resolved on a 25% PAGE/6 M urea/TBE gel. The band present in all lanes (above P + 1) was a contaminant produced during the synthesis of dinucleotide. The difference in total radioactivity among the lanes may be attributed to variations during ethanol precipitation of the short RNA products.

replicon RNAs were transfected into Huh-7 cells by electroporation along with the wild-type RNA and a replication-defective construct in which the catalytic motif C (GDD) was removed. To determine whether those modified replicons were capable of replicating in Huh-7 cells, total RNA was extracted at different time intervals (days 2, 3, and 4) from the transfected cells and the level of HCV RNA was quantified by using a real-time quantitative PCR method. Figure 4 shows the result of the transient replication assay, in which the levels of replicon RNA were normalized as fold increase over that of the GDD deletion mutant (ΔGDD). Replicon RNA carrying substitutions K_{155}A (motif F), R_{168}A (motif F), D_{225}N (motif A), R_{386}Q (thumb), and β-hairpin truncations failed to show a steady increase in HCV RNA signal over time. In fact, a decline in viral RNA signal was observed in most of these cases, likely due to the gradual degradation of the initial input RNA. This observation indicated that replicon RNAs carrying these mutations did not replicate effectively or replicated at much lower levels than that of the wild-type replicon (NK5.1). Interestingly, replicon RNA containing the conservative R_{345}K substitution in motif D replicated very efficiently. This modified RNA reproducibly replicated to levels several folds higher than that of the wild-type NK5.1, demonstrating that this substitution can significantly improve the replication efficiency of the HCV replicon.

The difference in replication capability of the NS5B-modified replicon RNAs was further confirmed by comparing their colony formation efficiencies. In this case, transfected Huh-7 cells were under G418 selection for about two weeks after which drug-resistant colonies were visualized by staining. As shown in Fig. 5, unlike the

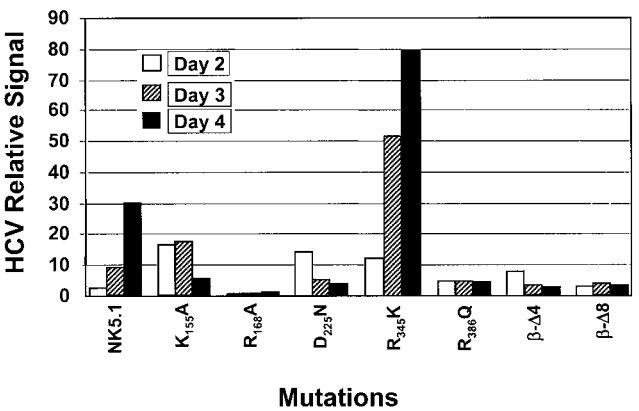

FIG. 4. Transient replication assay of HCV subgenomic replicon containing NS5B modifications. Wild-type (NK5.1) (Krieger *et al.,* 2001) and various replicon RNAs were transcribed *in vitro* by using bacteriophage T7 RNA polymerase. The transcripts were electroporated into Huh-7 cells and total cellular RNAs were extracted at different intervals (days 1, 2, and 3) after the transfection. HCV replicon RNA was analyzed by real-time quantitative RT-PCR (Perkin–Elmer) and normalized to a cellular mRNA (GAPDH). The signals represent fold increases of each replicon RNA over that of a replication-incompetent replicon in which the catalytic motif C (GDD) in NS5B was deleted (ΔGDD).

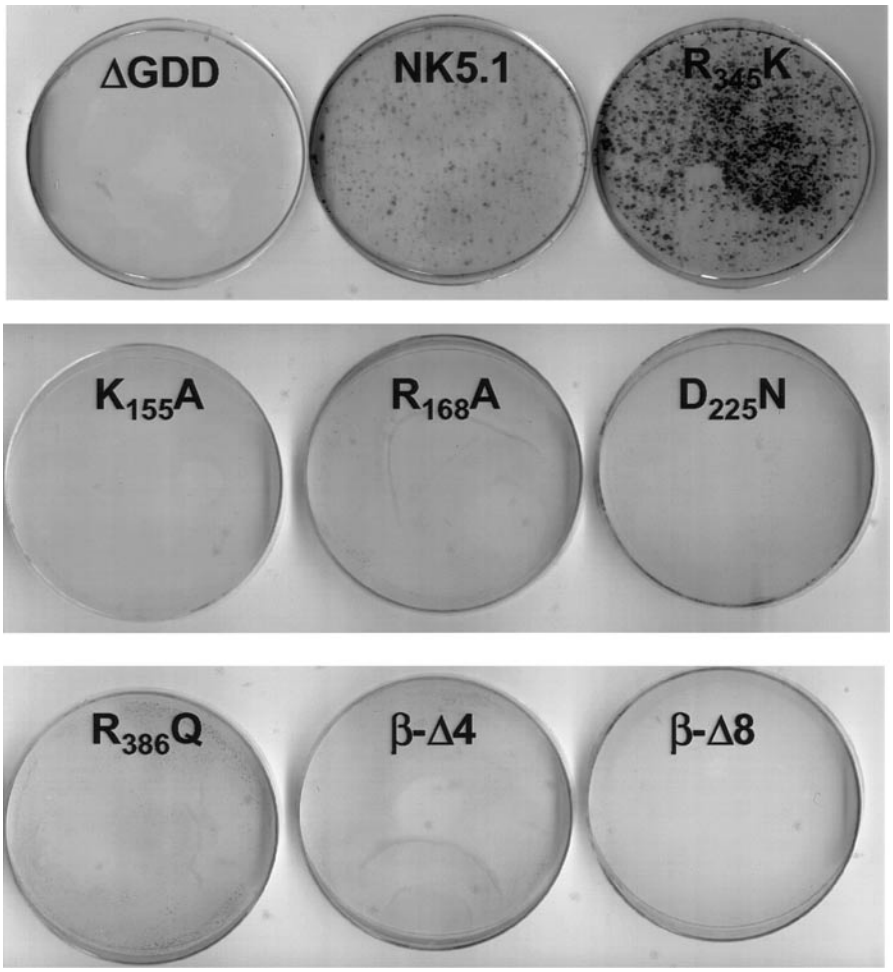

FIG. 5. Colony formation assay of the modified replicon RNAs. Huh-7 cells were transfected with individual replicon RNA as indicated. The cells were selected in the presence of G418 at 500 μg per ml for about two weeks after which the colonies were stained by Coomassie brilliant blue. ΔGDD is a replication-incompetent replicon that contains a deletion in motif C of NS5B.

wild-type NK5.1 that produced numerous drug-resistant colonies, the modified replicon RNAs, with the exception of that with the $R_{345}K$ substitution in motif D, produced no colonies. Replicon RNA with this conservative substitution produced colonies several folds (>4) more than that of NK5.1. These data indicated that the majority of the amino acid substitutions ($K_{155}A$, $R_{168}A$, $D_{225}N$, and $R_{386}Q$) rendered the replicon RNA incapable of replicating, or unable to replicate to significantly high levels to protect the cells from G418-mediated killing, due to their detrimental effects on the polymerase activity. On the other hand, the $R_{345}K$ substitution, while slightly increasing the enzymatic activity of NS5B, significantly improved the replication efficiency of the replicon RNA both in the transient replication assay and in the colony formation assay.

Interestingly, replicon RNA carrying the β-hairpin truncations was incapable of producing drug-resistant colonies despite repeated efforts. As shown in Fig. 2, NS5B proteins with such truncations possessed significant higher enzymatic activity than the wild-type protein. The replication assay clearly demonstrated that the β-hairpin plays an essential role(s) in viral RNA replication in the cell culture system but is not necessary for NS5B enzymatic activity *in vitro*.

DISCUSSION

Virally encoded polymerases play a central role in the replication of viral genomes. In HCV, NS5B protein has been shown to possess the RdRp activity in both primer-dependent and primer-independent (*de novo*) fashions (Behrens *et al.,* 1996; De Francesco *et al.,* 1996; Ferrari *et al.,* 1999; Hong *et al.,* 2001; Lohmann *et al.,* 1997, 1998; Luo *et al.,* 2000; Oh *et al.,* 1999, 2000; Sun *et al.,* 2000; Zhong *et al.,* 2000a,b). HCV NS5B contains six conserved sequence motifs characteristic of all RdRps. Crystal structural comparison between HCV NS5B and poliovirus $3D^{pol}$ and HIV RT also revealed a unique structural element, the β-hairpin, which exists only in HCV NS5B. Our previous enzymatic studies have suggested that this β-hairpin may play an important role in *de novo* initiation

of RNA replication. Presence of the β-hairpin in NS5B may ensure initiation of RNA synthesis from the 3′ terminus of the template RNA by properly positioning the terminus to the active site, a function thought to be critical for RdRps that use a *de novo* mechanism for initiation of RNA synthesis (Hong *et al.,* 2001).

A limited mutational analysis has been performed on several of the sequence motifs (motifs A to D) in HCV NS5B and has confirmed their functional importance for the polymerase activity (Lohmann *et al.,* 1997). However, the correlation between *in vitro* NS5B enzymatic activity and viral RNA replication in cell culture has not been analyzed due to the lack of a reliable cell culture system for HCV. In addition, our knowledge about the additional sequence or structural elements in HCV NS5B (e.g., motif F and β-hairpin) is still lacking. With the establishment of the HCV subgenomic replicon system, it has now become feasible to address some of these issues. In this study, we focused our efforts on a number of key residues in the conserved motifs that include motif F (K_{155}, R_{168}), motif A (D_{225}), motif D (R_{345}), a conserved arginine residue (R_{386}) in the thumb subdomain, and the β-hairpin. Our results clearly demonstrated that substitutions of most of these residues (K_{155}A, R_{168}A, D_{225}N, R_{386}Q) are detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells, confirming the essential functions of these motifs or residues.

In agreement with our previous article (Hong *et al.,* 2001), no reduction in polymerase activity was observed when the β-hairpin was shortened by four or eight amino acid residues. Instead, a significant enhancement in both primer-dependent and *de novo* initiation activity was observed. However, when these truncations were introduced into the replicon RNA, it completely lost the ability to replicate in Huh-7 cells. This result indicates that the β-hairpin is essential for viral RNA replication in the cell culture but is not required for the enzymatic activity of NS5B. We did however notice certain changes in the enzymatic properties of NS5B as the result of the truncations, in particular, the increased efficiency in utilizing stable duplex RNA as template and in initiating RNA synthesis from the internal positions of the template RNA (Hong *et al.,* 2001). These subtle changes could likely be responsible for the incompetent replication phenotype. It is conceivable that initiation from internal rather than terminal positions of the viral RNA template would result in production of defective RNA molecules that could interfere with the replication process. Alternatively, the β-hairpin may play additional roles in viral RNA replication, such as involvement in modulating the conformational changes of the polymerase protein during various steps of the replication process, or affecting the interaction between NS5B and other viral or host proteins that are part of the viral replicase complex. However, these possibilities cannot be addressed without detailed three-dimensional structural information or an *in vitro* system in which active replicase complex can be reconstituted from individual component proteins.

Another interesting observation involves the arginine residue in motif D, which when changed to a conservative lysine residue (R_{345}K) showed drastic improvements in both transient RNA replication and colony formation efficiencies over the wild-type replicon. This conservative change was shown previously (also as shown in Fig. 3) (Lohmann *et al.,* 1997) to increase NS5B RdRp activity by ~50% *in vitro*. This arginine residue is conserved in NS5B of all HCV isolates; however, an invariant lysine residue was found at this position in all RTs and RdRps (except that of HCV). As inferred from the three-dimensional structure of HIV RT, this lysine, together with the highly conserved carboxylates of motifs A and C, is directly involved in catalysis (Joyce and Steitz, 1995). We cannot explain why an arginine residue, which reduced both NS5B enzymatic activity *in vitro* and viral RNA replication efficiency in Huh-7 cells, is present at this position in all HCV isolates. Nonetheless, the finding is consistent with the low replication levels of HCV *in vivo*. The low replication levels may be necessary for the virus to evade the host immune surveillance and establish persistent infection in the infected humans. As a known fact, HCV establishes chronic infections in a majority (~80%) of infected individuals. However, it should be noted that the replicon system used in this study is not an authentic HCV cell culture system and only part of the viral replication cycle (i.e., RNA synthesis) can be studied. Therefore, this observation needs further confirmation with more authentic HCV replication culture systems, while its potential implication in the establishment of chronic/persistent viral infection may be addressed in suitable animal models *in vivo*.

MATERIALS AND METHODS

Cells

The Huh-7 was obtained from Ralf Bartenschlager (Mainz, Germany) and was identical to that described previously (Lohmann *et al.,* 1999). These cells were routinely grown in DMEM media (Gibco/BRL) supplemented with 10% FBS, 2 mM L-glutamine, nonessential amino acids, 100 U per ml of penicillin, and 100 μg/ml of streptomycin. Growth of HCV replicon-containing cells required addition of G418 to a final concentration of 500 μg per ml of the medium (Gibco/BRL). Cells were maintained in a subconfluent state to ensure optimal cell growth and hence efficient replicon replication.

Plasmids

DNA plasmid pNK5.1 (Krieger *et al.,* 2001) was used as the parental HCV replicon construct and is a derivative of the original pFK-I389neo/NS3–3′/wt clone described previously (Krieger *et al.,* 2001; Lohmann *et al.,* 1999). NS5B

mutants were constructed within the pNK5.1 background by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). The following mutations in NS5B were constructed: $K_{155}A$ (aag to gcg), $R_{168}A$ (cgt to gct), $D_{225}N$ (gac to aac), $R_{345}K$ (aga to aaa), and $R_{386}Q$ (cgt to caa). The β-hairpin truncation mutants β-Δ4 and β-Δ8 were designed and constructed as previously described (Hong *et al.,* 2001). Sequence of all mutant NS5B constructs was confirmed by a combination of Big Dye (ABI) PCR thermocycling and automated DNA sequencing (ABI377; Perkin–Elmer, Foster City, CA). Plasmid pET21b (Novagen, Madison, WI) was used to express NS5B proteins.

NS5B protein purification

NS5B polymerase protein was purified as described previously (Ferrari *et al.,* 1999; Lesburg *et al.,* 1999). All NS5B proteins were expressed with a 21 amino acid C-terminal truncation to enhance overall solubility. A C-terminal polyhistidine tag was also added to create fusion proteins for Ni-NTA column purification. Briefly, expression of NS5B proteins was achieved by transformation of pET21b-NS5B into the bacterial *Escherichia coli* host BL21 (DE3). Successful transformants were grown in Terrific broth (Gibco/BRL) and were induced by the addition of 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) for 4 h at 24°C. Cells were pelleted, resuspended in buffer (50 mM HEPES, pH 8, 0.6 M NaCl, 20% glycerol, and 0.1% octyl-β-glycoside), blended, and then microfluidized at 7500 psi for three cycles. Cell lysates were ultracentrifuged at 100,000 *g* for 1 h using a Type Ti 45 rotor and then the supernatant was passed through a 0.22-μm filter. Further purification was achieved by chromatography first using a chelating Sepharose Fast Flow column charged with nickel and then an SP Sepharose Fast Flow column. Proteins eluted from the SP column were applied to an Amicon Centriprep spin column to give a final concentration of approximately 10 mg/ml. Concentrated NS5B proteins were run on 10% SDS–PAGE gels for a visual assessment and were estimated to be over 90% pure.

In vitro enzymatic assays

NS5B RdRp activity was assessed by two different assay formats. (i) Primer-dependent elongation assay. In this assay, homopolymeric poly(A) RNA was used as template and oligo$(dT)_{12}$ as primer to measure NS5B-directed [α-^{33}P]UTP incorporation. Each reaction contains 50 mM HEPES, pH 7.3, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 10 μM UTP/10 μCi [α-^{33}P]UTP, 200 ng poly(A)/25 ng oligo$(dT)_{12}$, and 300 ng NS5B. The reaction was carried out at 30°C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. The labeled reaction products were resolved in a 15% PAGE/6 M urea/TBE gel and subjected to phosphorimaging. (ii) *De novo* initiation assay. In this assay, a synthetic oligoribonucleotide (5′ AAAAAAAAGC 3′) was used as template to direct initiation of *de novo* RNA synthesis from GTP to form the radiolabeled pppGpC dinucleotide product. Each reaction contained 50 mM HEPES, pH 7.3, 10 mM β-mecaptoethanol, 5 mM $MgCl_2$, 100 μM GTP, 100 μM CTP/10 μCi [α-^{33}P]CTP, 5–10 μM RNA template, and 300–500 ng NS5B protein. The reaction was carried out at 30°C for 30 min after which the products were resolved on a 25% PAGE/6 M urea/TBE gel. Products were detected by autoradiography. Other RNA templates and primers were also used in the RdRp assays as indicated in Fig. 3.

In vitro RNA transcription, electroporation, and selection of G418-resistant Huh-7 cells

HCV replicon plasmids were sequentially digested with *Ase*I and *Sca*I restriction enzymes, extracted with phenol/chloroform, and then precipitated with isopropanol. Two and a half micrograms of linearized DNA was transcribed into RNA using the T7-MEGAscript kit from Ambion (Austin, TX) according to the manufacturer's instructions. Transcribed RNA was DNase I digested for 45 min at 37°C, extracted with phenol/chloroform, and precipitated with isopropanol. Yield of the transcript RNAs was determined by measurement of the optical density at 260 nm. Integrity of the RNA was checked by agarose gel electrophoresis. Subconfluent parental Huh-7 cells were prepared for electroporation by methods described previously (Krieger *et al.,* 2001; Lohmann *et al.,* 1999). Briefly, 4 million Huh-7 cells were mixed with 0.5–1 μg replicon RNA and 9 μg carrier RNA (total yeast RNA) and electroporated on a Bio-Rad Gene Pulser II at 270 V, 960 μF, at maximum resistance, in a cuvette with a 0.4-cm gap. Electroporated cells were plated in DMEM growth medium and, if drug selection was required, changed to medium containing 500 μg per ml of G418 24 h after initial plating. Medium for selecting cells was changed twice weekly. Drug-resistant colonies were fixed and then stained with Coomassie brilliant blue approximately 2 weeks postelectroporation.

Transient replication assay and quantitative PCR analysis

HCV replicon RNA was electroporated into parental Huh-7 cells and maintained in DMEM growth medium in the absence of G418. Cells were harvested at different intervals (days 2, 3, and 4) after electroporation and total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. RNAs were quantitated by spectrophotometry (A_{260nm}) and then 50 ng were used in quantitative RT-PCR reactions (Taqman One Step Master Mix, Applied Biosystems) with probes and primers specific for HCV 5′UTR and GAPDH mRNA as listed as follows: GAPDH probe: 5′-VIC-accag-

gcgcccaatacgaccaa-TAMRA-3′; GAPDH PCR primers: 5′-tgaaggtcggagtcaacgg-3′ forward; 5′-cagagttaaaagcagccctggt-3′ reverse; HCV 5′UTR probe: 5′-FAM-tgccccgggagtctcgtagacc-TAMRA-3′; HCV PCR primers: 5′-agtagtgttgggtcgcgaaag-3′ forward; 5′-tgaggtttaggattcgtgctcat-3′ reverse. Cycle threshold (CT) values for amplified RT/PCR products were obtained using the ABI 7700 Sequence Detector.

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REFERENCES

Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., and Miyano, M. (1999). Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Struct. Fold Des.* **7,** 1417–26.

Bartenschlager, R., and Lohmann, V. (2000). Replication of hepatitis C virus. *J. Gen. Virol.* **81,** 1631–1648.

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V., and Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30,** 316–624.

Behrens, S.-E., Tomei, L., and De Francesco, R. (1996). Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* **15,** 12–22.

Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290,** 1972–1975.

Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R. L., Mathieu, M., De Francesco, R., and Rey, F. A. (1999). Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **96,** 13034–13039.

Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-born non-A, non-B viral hepatitis genome. *Science* **244,** 359–364.

De Francesco, R., Behrens, S. E., Tomei, L., Altamura, S., and Jiricny, J. (1996). RNA-dependent RNA polymerase of hepatitis C virus. *Methods Enzymol.* **275,** 58–67.

Ferrari, E., Wright-Minogue, J., Fang, J. W. S., Baroudy, B. M., Lau, J. Y. N., and Hong, Z. (1999). Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. *J. Virol.* **73,** 1649–1654.

Hong, Z., Beaudet-Miller, M., Lanford, R. E., Guerra, B., Wright-Minogue, J., Skelton, A., Baroudy, B. M., Reyes, G. R., and Lau, J. Y. N. (1999). Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. *Virology* **256,** 36–44.

Hong, Z., Cameron, C. E., Lau, J. Y. N., and Zhong, W. (2001). Hepatitis C virus NS5B polymerase employs a novel mechanism to ensure terminal initiation during replication. *Virology* **285,** 6–11.

Houghton, M. (1996). Hepatitis C viruses. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 3rd ed., pp. 1035–1058. Raven Press, New York.

Joyce, C. M., and Steitz, T. A. (1995). Polymerase structures and function: Variations on a theme? *J. Bacteriol.* **177,** 6321–6329.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M., and Rice, C. M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* **277,** 570–574.

Krieger, N., Lohmann, V., and Bartenchlager, R. (2001). Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J. Virol.* **75,** 4614–4624.

Lesburg, C. A., Cable, M. B., Ferrari, E., Hong, Z., Mannarino, A. F., and Weber, P. C. (1999). Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat. Struct. Biol.* **6,** 937–943.

Lohmann, V., Korner, F., Dobierzewska, A., and Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* **73,** 1437–1449.

Lohmann, V., Korner, F., Herian, U., and Bartenschlager, R. (1997). Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* **71,** 8416–8428.

Lohmann, V., Korner, F., Koch, J.-O., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285,** 110–113.

Lohmann, V., Roos, A., Korner, F., Koch, J. O., and Bartenschlager, R. (1998). Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *Virology* **249,** 108–118.

Luo, G., Hamatake, R. K., Mathis, D. M., Racela, J., Rigat, K. L., Lemm, J., and Colonno, R. J. (2000). *De novo* initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. *J. Virol.* **74,** 851–863.

Oh, J. W., Ito, T., and Lai, M. M. (1999). A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J. Virol.* **73,** 7694–7702.

Oh, J. W., Sheu, G. T., and Lai, M. M. (2000). Template requirement and initiation site selection by hepatitis C virus polymerase on a minimal viral RNA template. *J. Biol. Chem.* **275,** 17710–17717.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., and Bartenschlager, R. (2001). Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J. Virol.* **75,** 1252–1264.

Rice, C. M. (1996). Flaviviridae: The viruses and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 3rd ed., pp. 931–960. Raven Press, New York.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, T. Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M., and Kuo, G. (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Sci. Acad. USA* **87,** 6547–6549.

Seeff, L. B. (1999). Natural history of hepatitis C. *Am. J. Med.* **107,** 10S–15S.

Sun, X.-L., Johnson, R. B., Hockman, M. A., and Wang, Q. M. (2000). De novo RNA synthesis catalyzed by HCV RNA-dependent RNA polymerase. *Biochem. Biophys. Res. Commun.* **268,** 798–803.

World Health Organization. (1996). Hepatitis C: Seroprevalence of hepatitis C virus (HCV) in a population sample. *Wkly. Epidemiol. Rec.* **71,** 346–349.

Yanagi, M., Purcell, R. H., Emerson, S. U., and Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc. Natl. Acad. Sci. USA* **94,** 8738–8743.

Yanagi, M., St. Claire, M., Shapiro, M., Emerson, S. U., Purcell, R. H., and Bukh, J. (1998). Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. *Virology* **244,** 161–172.

Zhong, W., Ingravallo, P., Wright-Minogue, J., Uss, A. S., Skelton, A., Ferrari, E., Lau, J. Y., and Hong, Z. (2000a). Template/primer requirements and single nucleotide incorporation by hepatitis C virus nonstructural protein 5B polymerase. *J. Virol.* **74,** 9134–9143.

Zhong, W., Uss, A. S., Ferrari, E., Lau, J. Y., and Hong, Z. (2000b). De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. *J. Virol.* **74,** 2017–2022.